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Attorney Docket Number	KNAUTHE-08713

ENCLOSURES *(Check all that apply)*

- [x] Fee Transmittal Form
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Remarks

Submitted herewith, in triplicate, is Appellant's Brief in furtherance of the Notice of Appeal filed June 5, 2006 and received in the U.S. Patent Office on June 7, 2006. Applicants also submit the required fee under Section 1.17(h). Please charge any additional fees, including any fees necessary for extensions of time, or credit overpayment to Deposit Account No. 08-1290.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	MEDLEN & CARROLL, LLP		
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Date	August 7, 2006	Reg. No.	44,174

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PATENT
Attorney Docket No. **KNAUTHE-08713**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Gabrielle Multhoff *et al.*		
Serial No.:	09/646,835	Group No.:	1643
Filed:	01/11/01	Examiner:	Christopher Yaen
Entitled:	**Use of Hsp70 Proteins**		

APPELLANTS' BRIEF
APPEAL NO.:

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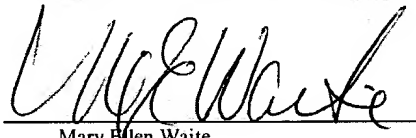
By: 
Mary Ellen Waite

Sir or Madam:

This Brief is in furtherance of the Notice of Appeal filed June 5, 2006.

The fees required under § 1.17(h) and any required Petition for Extension of Time for filing this Brief and fees therefore are dealt with in the accompanying TRANSMITTAL OF APPEAL BRIEF.

This Brief is transmitted in triplicate. [37 C.F.R. § 41.37(c)].

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This Brief contains these items under the following headings and in the order set forth below [37 C.F.R. § 41.37(c)]:

I. REAL PARTY IN INTEREST

The real party in interest is Dr. Gabriele Multhoff.

II. RELATED APPEALS AND INTERFERENCES

There are no known related appeals or interferences known to Appellants, Appellants' legal representative, or the Assignee.

III. STATUS OF CLAIMS

Claims 1 - 30 were filed in the original application, a National Phase Entry from PCT Application EP99/02165. During prosecution of the application, Claims 31 –87 were added, Claims 1-48, 50-60, and 78-82 cancelled, and claim 49 withdrawn. Claims 61-77 and 83-87 are currently pending and have been rejected by the Office in the Final Office Action dated April 4, 2006. Therefore, Claims 61-77 and 83-87 are pending in this appeal. No other claims are pending. Thus, Appellants appeal the Final Office Action of April 4, 2006. The Claims, as they now stand, are set forth in the Claims Appendix.

IV. STATUS OF AMENDMENTS

Appellants' Response to the Office Action mailed March 1, 2006 has been entered per the Final Office Action dated April 4, 2006. Amendments to the claims that were made in the March 1, 2006 Response were acknowledged in the Final Office Action dated April 4, 2006. Thus, there are no pending amendments not entered into the record.

V. SUMMARY OF CLAIMED SUBJECT MATTER

This invention relates to the use of Hsp70 polypeptides to activate Natural Killer Cells (NK-cells) ex vivo. See, English Translation of PCT Application at 1. In particular, the invention relates to the use of a protein, protein fragment, or polypeptide selected from the group consisting of a Hsp70 protein of SEQ ID NO.: 1, a C-terminal fragment of Hsp70, wherein said fragment comprises amino acids 384-641 of SEQ ID NO.: 1, and a polypeptide having 70% or greater homology to amino acids 384-641 of SEQ ID NO.: 1. Id. at 2-3. Preferably, the protein, fragment, or polypeptide induce an immune response by the treated NK cells. Id. at 5. Preferably, the immune response increases cytolytic activity of the NK cells or stimulates proliferation of the NK cells. Id. The Hsp70 polypeptides are added to a physiological suspension containing NK cells, such as a peripheral mononuclear blood cell fraction. Id. at 14, Example 1. The cell suspension can contain diseased cells, including leukemia cells, lymphoma cells, tumor cells, metastasizing cells of solid tumors and cells from a virally, mycotically and/or bacterially infected patient. Id. at 5, 15, Example 2. The NK-cells are thereby induced to attack Hsp70 expressing diseased cells, such as tumor cells. Id. at 15-18, examples 2 and 3.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

There is one ground of rejection to be reviewed on appeal:

Issue 1 – Whether Claims 61-77 and 83-87 are supported by an adequate written description.

VII. ARGUMENT

Issue 1 - Claims 61-77 and 83-87 are supported by an adequate written description.

Claims 61-77 and 83-87 are rejected under 35 U.S.C. 112, first paragraph, as allegedly lacking an adequate written description. As explained below, the Examiner's rejection is unsupportable because 1) Eli Lilly and related cases are not controlling and 2) the Examiner has ignored the Written Description Guidelines promulgated by the Office.

1. Eli Lilly is not controlling

The Examiner's rejection relies heavily on Eli Lilly and related cases. Final Office Action dated April 4, 2006 at p. 3-4. The Examiner states that "the Federal Circuit held that a generic statement that defines a genus of nucleic acids by only their functional activity does not provide an adequate written description of the genus." Id. at 3. The Examiner goes on to state "by analogy, a generic statement that defines a genus of polypeptides having 70% or greater homology to amino acids 384-641 of SEQ ID NO.:1 by only their common ability to induce an immune response by NK cells, wherein the response increases cytolytic activity of the NK cells or stimulates NK cell proliferation does not adequately describe the genus as a whole."

As a preliminary matter, *Eli Lilly* is not analogous as suggested by the Examiner. Neither the specification nor the claims of the patent addressed by the Federal Circuit in Eli Lilly contained the sequence for human insulin. Regents of the University of California v. Eli Lilly, 43 USPQ2d 1398 (Fed. Cir. 1997). In the instant, the sequence of a Hsp70 is provided in the both the specification and the claims and, as described in more detail below, was well known in the art as of the priority date of the application. Thus, applicants have not attempted to define a genus of nucleic acids by only their functional activity. Instead, applicants have disclosed and referred to specific sequences. Thus, the analogy the Examiner attempts to draw has no merit.

The Examiner next cites Noelle v. Lederman for the proposition that "the Federal Circuit has decided that a patentee of a biotechnological invention cannot necessarily claim a genus after only describing a limited number of species because there may be unpredictability in the results obtained from other species other than those specifically enumerated." Final Office Action at 4. However, Noelle dealt with an application in which the claims were directed to monoclonal antibodies and in which a only a single monoclonal antibody epitope was disclosed. Noelle v.

Lederman, 69 USPQ2d 1508 (Fed. Cir. 2004). Noelle did not address the situation where specific sequences are provided in the application and claims and known in the art. Thus, Noelle is not applicable to the instant case either.

The Examiner does admit that "Applicants have pointed to several species of HSPs that may fall within the genus of at least 70% homologous to amino acids 384-641 of SEQ ID NO:1." Final Office Action at 4. The Examiner also does not dispute, and simply dismisses the evidence provided by Applicants, that Milner and Campbell and Multhoff et al. teach that sequences within the claimed genus were known in the art. Id. at 3. In fact, the Examiner appears to accept that Milner and Campbell "teaches Hsp70-Hom and Hsp70-B both of which are at least 70% homologous to amino acids 384-641" and that Multhoff et al. "provides an overview of the Hsp70 multigene family." Id.

For the convenience of the Office, a copy of the Milner and Campbell reference is provided herewith. As demonstrated in Example 1 and shown in Fig. 1A of the present application, the proliferation and thus activity of NK cells could be stimulated by recombinant human Hsp70 as well as by the C-terminal fragment of homologous protein rHsp70homC (see, also, the Specification at page 16, line 2 through the completion of the second full paragraph).

Thus, the Applicants have actually reduced to practice a sequence that is 70% or greater in homology to amino acids 384-641 of SEQ ID NO: 1. Furthermore, the enclosed Milner and Campbell reference provides evidence that already in 1990, i.e. eight years prior to the effective filing date of the present application, several members of the Hsp70 family were known, which can be used in accordance with the teaching of the present invention.

For example, besides the Hsp70-Hom protein the Milner and Campbell reference discloses in Fig. 4 another homologous Hsp70 protein, i.e. Hsp70-B', the amino acid sequence of which has a homology of about 74% to amino acids 384-641 of the reference Hsp70 protein. Furthermore, reference is made to the publications by the inventor Multhoff et al., Cell Stress & Chaperones 1 (1996), 6-1 1 and Multhoff et al., Biol. Chem. 379 (1998), 295-300, both referenced at Page 1 of the present application, which provide an overview concerning Hsp70 multigene family including various citations. Thus, besides the fact that applicant actually reduced to practice homologous sequences of amino acids 384-641 of SEQ ID NO: 1 the prior art at the time the application was filed provided already a vast source of the genus of Hsp70 sequences that are encompassed in the claimed method.

In this respect, the Examiner's attention is respectfully directed to the Federal Circuit's recent holding in Falkner v. Inglis, 448 F.3d 1357; 79 U.S.P.Q.2D (BNA) 1001 (Fed. Cir. 2006). In that case, the Federal Circuit specifically held that "Eli Lilly does not set forth a per se rule that whenever a claim limitation is directed to a macromolecular sequence, the specification must always recite the gene or sequence, regardless of whether it is known in the prior art." Id. at 1367. The Federal Circuit went on to explain that:

> Thus, "[w]hen the prior art includes the nucleotide information, precedent does not set a per se rule that the information must be determined afresh." Id. at 1358. Rather, we explained that:
>
> > The descriptive text needed to meet these requirements varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence. The law must be applied to each invention that enters the patent process, for each patented advance is novel in relation to the state of the science. Since the law is applied to each invention in view of the state of relevant knowledge, its application will vary with differences in the state of knowledge in the field and differences in the predictability of the science.
> > Id. at 1357.
>
> Indeed, a requirement that patentees recite known DNA structures, if one existed, would serve no goal of the written description requirement. It would neither enforce the quid pro

> quo between the patentee and the public by forcing the disclosure of new information, nor would it be necessary to demonstrate to a person of ordinary skill in the art that the patentee was in possession of the claimed invention. As we stated in Capon, "[t]he 'written description' requirement states that the patentee must describe the invention; it does not state that every invention must be described in the same way. As each field evolves, the balance also evolves between what is known and what is added by each inventive contribution." Id. at 1358. Indeed, the forced recitation of known sequences in patent disclosures would only add unnecessary bulk to the specification.

Id. at 1367-68. The Federal Circuit then specifically held that "where, as in this case, accessible literature sources clearly provided, as of the relevant date, genes and their nucleotide sequences (here "essential genes"), satisfaction of the written description requirement does not require either the recitation or incorporation by reference (where permitted) of such genes and sequences." Id. In the instant case, sequences within the claims were known in the art and reference sequences were described in the specification and identified in the claims. That is all the written description that is needed.

Additionally, Applicants note that the priority date for the present application is March 27, 1998. The priority date for the patent addressed by the Federal Circuit in Eli Lilly was September 12, 1979 (i.e., U.S. Pat. No. 4,431,740). Regents of the Univ. of California v. Eli Lilly, 119 F.3d 1559, 1562 (Fed. Cir. 1997). As held by the Federal Circuit in Falkner, "Since the law is applied to each invention in view of the state of relevant knowledge, its application will vary with differences in the state of knowledge in the field and differences in the predictability of the science." Falkner, 448 F.3d at 1367-68. Surely, the state of art molecular biology had advanced considerably in the approximately 20 years elapsed between the filing the patent at issue in Eli Lilly and the present application. The simple fact is that the macromolecules utilized in the instant claims were both known when the application was filed and described in detail in the application itself. These sequences are representative of a broader genus, which any person of skill in the art can identify. Indeed, even if a person skilled in the art did not want to rely on the available Hsp70 protein species available at the time the present application was filed, the specification provides sufficient guidance how to produce and test appropriate derivatives of the exemplified Hsp70 protein and fragments thereof (see, e.g., the Specification at Page 3, second to fourth full paragraphs, Page 4, the paragraph bridging to Page 5 and Example 1 which describes spanning experiments for testing the capability of Hsp70 species to increase the proliferation of NK cells).

2. Application of the Written Description Guidelines compels allowance of the claims

Applicants respectfully refer the Examiner to the USPTO's "Synopsis of Application of Written Description Guidelines", and in particular to Example 14, pages 53-55. Consideration of the Examples in the Written Description Guidelines establishes that the claims are supported by an adequate written description.

The claim of Example 14 of the Written Description Guidelines recites a protein having SEQ ID NO:3 and variants thereof that are at least 95% identical to SEQ ID NO:3 and catalyze the reaction of A->B. The disclosure of Example 14 provides a single species (SEQ ID NO:3) that has actually been reduced to practice, and describes an assay for identifying the variants having the desired catalytic activity. The analysis considers (1) whether the members of genus vary substantially from each other; and (2) whether the disclosed species is representative of the members of the genus; in order to determine whether one of skill in the art would determine if the applicant was in possession of the necessary common attributes possessed by the members of the genus.

For Example 14, it was determined that the member species did not substantially vary since the variants have 95% identity or greater to the reference sequence, and also possess the catalytic activity. It was also determined that the disclosed species was representative since all members must have at least 95% structural identity to SEQ ID NO:3. The analysis determined that one of skill in the art would conclude that the applicant was in possession of the necessary common attributes possessed by the members of the genus, and therefore the disclosure in this Example meets the written description requirement.

Applicants submit that the instant claims can be analyzed in a similar manner to that provided in Example 14. First, the polypeptides do not substantially vary as members of a genus since they all have at least 70% homology to SEQ ID NO:1 and must possess the activity of inducing an immune response by NK cells. Second, the polypeptide having SEQ ID NO:1 is a representative species of the genus since all polypeptides must have at least 70% homology to this sequence. Therefore, one of skill in the art would conclude that the Applicants were in possession of the necessary common attributes possessed by the members of the genus, and therefore the instant specification meets the written description requirement for these claims.

The Examiner seems to attempt to draw a distinction between the 70% limitation in the present claims and the 95% homology limitation in Example 14. However, this distinction is without meaning. The Written Description Guidelines focus on the provision of a reference sequence and a limitation as to function. Both of these limitations are present in the instant claims. As a result, the Written Description Guidelines are persuasive authority that the instant claims are allowable.

VIII. CLAIMS APPENDIX

1-48. (Cancelled)

49. (Withdrawn) The method of claim 43, wherein said infectious diseases are viral, mycotic or bacterial diseases.

50-60. (Cancelled)

61. (Previously presented) A method for the ex vivo activation of NK- cells, comprising: contacting NK cells in physiological suspension with an isolated and uncomplexed protein, protein fragment, or polypeptide selected from the group consisting of a Hsp70 protein of SEQ ID NO.: 1, a C-terminal fragment of Hsp70, wherein said fragment comprises amino acids 384-641 of SEQ ID NO.: 1, and a polypeptide having 70% or greater homology to amino acids 384-641 of SEQ ID NO.: 1, wherein said isolated protein, fragment, or polypeptide induce an immune response by NK cells, and further said response increases cytolytic activity of the NK cells or stimulates proliferation of the NK cells.

62. (Previously presented) The method of claim 61, wherein said activation of said cells further comprises stimulation of proliferation and/or an increase in cytotoxicity.

63. (Previously presented) The method of claim 61, wherein said physiological suspension containing NK cells comprises a peripheral mononuclear blood cell fraction or fractions thereof.

64. (Previously presented) The method of claim 61, wherein said suspension further comprises cells expressing cell-surface Hsp70.

65. (Previously presented) The method of claim 64, wherein said expressing cells comprise diseased cells from a patient.

66. (Previously presented) The method of claim 65, wherein said diseased cells are selected from the group consisting of leukemia cells, lymphoma cells, tumor cells, metastasizing cells of solid tumors and cells from a virally, mycotically and/or bacterially infected patient.

67. (Previously presented) The method of any one of Claims 61-66, wherein said contacting is carried out for at least 3 hours.

68. (Previously presented) The method of claim 67, wherein said contacting is carried out for 4 days.

69. (currently amended) The method of claim 67, wherein ~~said conditions~~ said contacting further comprise addition of cytokine.

70. (Previously presented) The method of claim 69, wherein the cytokine is an interleukin.

71. (Previously presented) The method of claim 70, wherein said interleukin is selected from the group consisting of IL-2, IL-12 and IL-15.

72. (Previously presented) A method for the in vivo activation of the immune system in a patient in need thereof comprising:

i) administering to said patient a pharmaceutically effective amount of NK cells obtained by the method of claim 61; and

ii) optionally administering to said patient, concurrently or subsequently, a pharmaceutically effective amount of an isolated and uncomplexed protein, protein fragment, or polypeptide selected from the group consisting of a Hsp70 protein of SEQ ID NO: 1, a C-terminal fragment of Hsp70, wherein said fragment comprises amino acids 384-641 of SEQ ID NO.: 1, and a polypeptide having 70% or greater homology to amino acids 384-641 of SEQ ID NO.: 1, wherein said isolated protein, fragment, or

polypeptide induces an immune response by NK cells, and wherein said response increases cytolytic activity of the NK cells or stimulates proliferation of the NK cells.

73. (Previously presented) The method of claim 72, where said patient is suffering from a disease selected from the group consisting if cancerous, infectious and autoimmune disease.

74. (Previously presented) The method of claim 72, further comprising administering a cytokine.

75. (Previously presented) The method of claim 74, wherein said cytokine is an interleukin.

76. (Previously presented) The method of claim 75, wherein said interleukin is selected from the group consisting of IL-2, IL-12 and IL-15.

77. (Previously presented) The method of claim 73, wherein said cancerous disease is selected from the group consisting of tumors, solid tumors, metastic tumors, leukemias and lymphomas.

78-82. (Canceled).

83. (Previously presented) A method for in vivo activation of the immune system in a patient in need thereof comprising administering to said patient a pharmaceutically effective amount of an isolated and uncomplexed protein, protein fragment, or polypeptide selected from the group consisting of a Hsp70 protein of SEQ ID NO.:1, a C-terminal fragment of Hsp70, wherein said fragment comprises amino acids 384-641 of SEQ ID NO.: 1, and a polypeptide having 70% or greater homology to amino acids 384-641 of SEQ ID NO.: 1; wherein said isolated protein, fragment, or polypeptide induces an immune response by NK cells, and wherein

said response increases cytolytic activity of the NK cells or stimulates proliferation of the NK cells.

84. (Previously presented) The method of claim 83, where said patient is suffering from a disease selected from the group consisting of cancerous, infectious and autoimmune disease.

85. (Previously presented) The method of claim 83, further comprising administering a cytokine.

86. (Previously presented) The method of claim 85, wherein said cytokine is an interleukin.

87. (Previously presented) The method of claim 86, wherein said interleukin is selected from the group consisting of IL-2, IL-12 and IL-15.

IX. EVIDENCE APPENDIX

Per 37 C.F.R. §41.37(c)(ix), a copy of Milner and Campbell, Structure and expression of the three MHC-linked HSP70 genes, Immunogenetics 32:242-251 (1990) is provided with the present appeal brief.

X. RELATED PROCEEDINGS APPENDIX

There are no related proceedings.

XI. CONCLUSION

For the foregoing reasons, it is submitted that the Office's rejection of Claims 61-77 and 83-87 is erroneous, and reversal of the rejection is respectfully requested. Appellant requests either that the Board render a decision as to the allowability of the claims, or alternatively, that the application be remanded for reconsideration by the Office.

Dated: August 7, 2006

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Structure and expression of the three MHC-linked *HSP70* genes

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Abstract. A duplicated locus encoding the major heat shock-induced protein HSP70 is located in the major histocompatibility complex (MHC) class III region 92 kilobases (kb) telomeric to the *C2* gene. Nucleotide sequence analysis of the two intronless genes, *HSP70-1* and *HSP70-2*, has shown that they encode an identical protein product of 641 amino acids. A third intronless gene, *HSP70-Hom*, has also been identified 4 kb telomeric to the *HSP70-1* gene. This encodes a more basic protein of 641 amino acids which has 90% sequence similarity with HSP70-1. In order to investigate the expression of the three (MHC)-linked *HSP70* genes individually by northern blot analysis, we have isolated locus-specific probes from the 3′ untranslated regions of the genes. The *HSP70-1* and *HSP70-2* genes have been shown to be expressed at high levels as a ~2.4 kb mRNA in cells heat-shocked at 42 °C. *HSP70-1* is also expressed constitutively at very low levels. The *HSP70-Hom* gene, which has no heat shock consensus sequence in its 5′ flanking sequence, is expressed as a ~3 kb mRNA at low levels both constitutively and following heat shock.

Introduction

Heat shock proteins, or stress proteins, are expressed in response to heat shock and a variety of other stress stimuli including oxidative free radicals and toxic metal ions (for reviews see Lindquist 1986; Lindquist and Craig 1988). This response has been observed in all species examined to date. The family of stress proteins of M_r 70000, HSP70, is probably the most predominant and is highly conserved throughout evolution. The amino acid sequence similarities between eukaryotic HSP70s range from 60%-78% and there is 47% similarity between human HSP70 and the *Escherichia coli* homologue *dnaK* (Hunt and Morimoto 1985).

Two independent studies (Goate et al. 1987; Harrison et al. 1987) using somatic cell hybrid analysis and genomic Southern blotting to detect restriction fragment length polymorphisms have demonstrated the presence of multiple autosomal loci for HSP70 in the human genome. These were shown to lie on chromosomes 6, 14, 21, and at least one other autosome. The chromosome 6 *HSP70* loci were localized to a region on the short arm close to the major histocompatibility complex (MHC). During characterization of the class III region of the MHC for additional loci (Sargent et al. 1989a, b) a duplicated locus encoding HSP70 was found between the complement and tumor necrosis factor (*TNF*) genes (Fig. 1A). The *HSP70* loci are 12 kilobases (kb) apart and lie 92 kb telomeric of the *C2* gene. In addition, a region of similarity was localized to a segment of DNA lying ~4 kb telomeric to the first copy of the duplicated *HSP70* locus. *HSP70* loci have also been mapped to the equivalent region of the rat (Wurst et al. 1989) and caprine (Cameron et al. 1990) MHCs.

The human *HSP70* multigene family encodes several highly conserved proteins with structural and functional properties in common, but which vary in their inducibility in response to metabolic stress. The structure of two of these has been completely determined (Wu et al. 1985; Hunt and Morimoto 1985; Leung et al. 1990). The HSP70 sequence determined by Hunt and Morimoto (1985) is encoded by one of the genes (*HSP70-1*) located in the MHC (Harrison et al. 1987; Sargent et al. 1989b). This sequence shares 77% similarity with a more basic HSP70 protein, HSP70B′ (Leung et al. 1990). Other members of the HSP70 protein family include a clathrin uncoating ATPase of M_r 72000 (Chappell et al. 1986) and a glucose-regulated protein (GRP78) of M_r 78000 (Ting and Lee 1988). In addition to the stress-induced proteins, the human genome also contains at least one *HSP70* cognate

The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the accession numbers M34267-9.

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gene (*HSC70*; Dworniczak and Mirault 1987) which is constitutively expressed and shares 82% similarity in its amino acid sequence with *HSP70*.

In order to define the structure of the three MHC-linked *HSP70* loci and to determine whether they were identical to previously identified members of the *HSP70* family, we determined their nucleotide sequences. In addition, we have isolated locus-specific probes from the 3′ untranslated regions of the three genes in order to investigate their expression both constitutively and in response to heat shock.

Materials and methods

Cloning and nucleotide sequence analysis. A 5.6 kb *Eco* RI/*Hind* III fragment containing the *HSP70-1* gene and a 3.6 kb *Pvu* II fragment containing the *HSP70-2* gene were isolated from cos H92 and cos 181 (Sargent et al. 1989a), respectively (Fig. 1B). These fragments were cloned into *Pvu* II-cut pATX and designated pHR-5.6 and pP-3.6, respectively. A 2.4 kb *Bam* HI/*Hind* III fragment containing the coding sequence and 3′ untranslated region of *HSP70-1* and a 0.4 kb *Bam* HI/*Nco* I fragment containing the 5′ flanking and 5′ untranslated region of this gene were isolated from clone pHR-5.4. These were subcloned into *Bam* HI/*Hind* III and *Bam* HI/*Hinc* II-cut Bluescript KS$^+$ vector (clones BS2.4-1 and BS0.4-1). Similarly, a 2.6 kb *Bam* HI/*Pvu* II fragment containing the coding and 3′ untranslated sequence of *HSP70-2* and a 0.9 kb *Bam* HI/*Pvu* II fragment encompassing the 5′ flanking and 5′ untranslated sequence were isolated from pP-3.6 and cloned into *Bam* HI/*Hinc* II-cut Bluescript vector (clones BS2.6-2 and BS0.9-2). The homologous region was isolated from cos H92 in two overlapping fragments: a 4 kb *Bam* HI/*Nco* I fragment which was cloned into *Bam* HI/*Hinc* II-cut Bluescript SK$^+$ (BS4.0-H), and a 1.3 kb *Bam* HI/*Hind* III fragment which was cloned into Bluescript KS$^+$ (BS1.3-H; Fig. 1B). Some smaller fragments from the homologous region were further subcloned into *Sma* I-cut M13 mp10. Single-stranded DNA was recovered from the Bluescript or M13 subclones and sequenced by the dideoxy nucleotide chain termination method using Sequenase (US Biochemicals, Cleveland, Ohio). In the case of the Bluescript clones the helper phage M13K07 was used for ssDNA recovery, in the presence of kanamycin. Nucleotide sequence was obtained using the M13 universal primer or specific oligonucleotide primers, which in the case of the *HSP70-1* and *HSP70-2* genes were based on the sequence published by

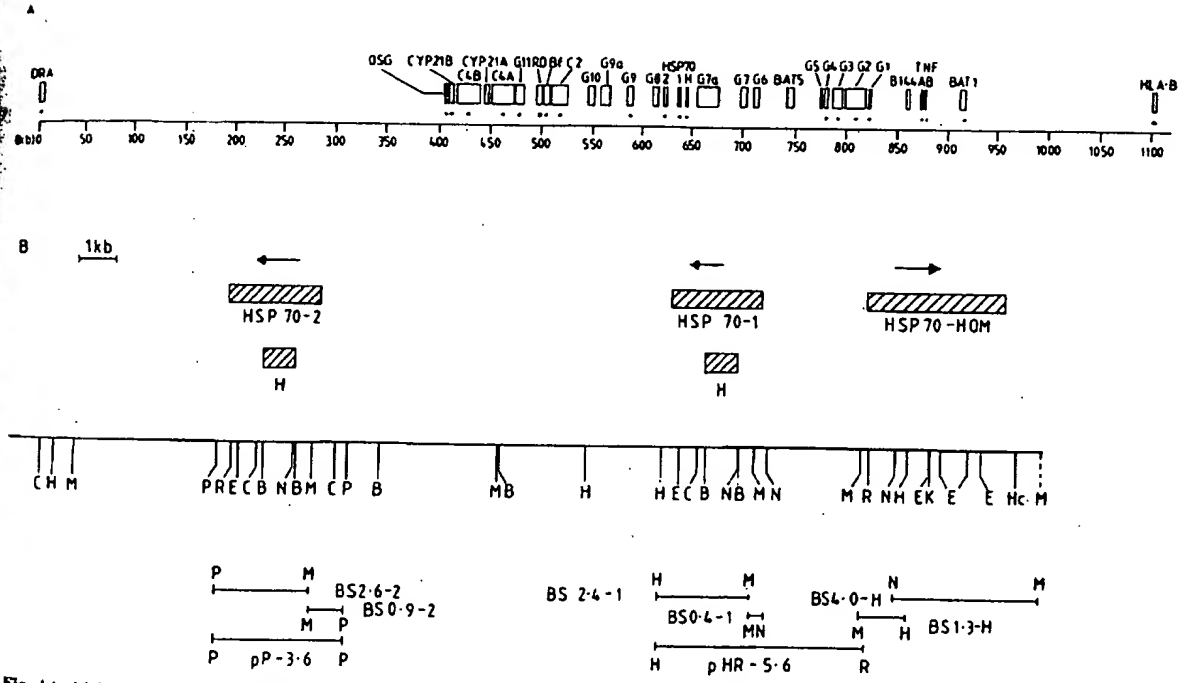
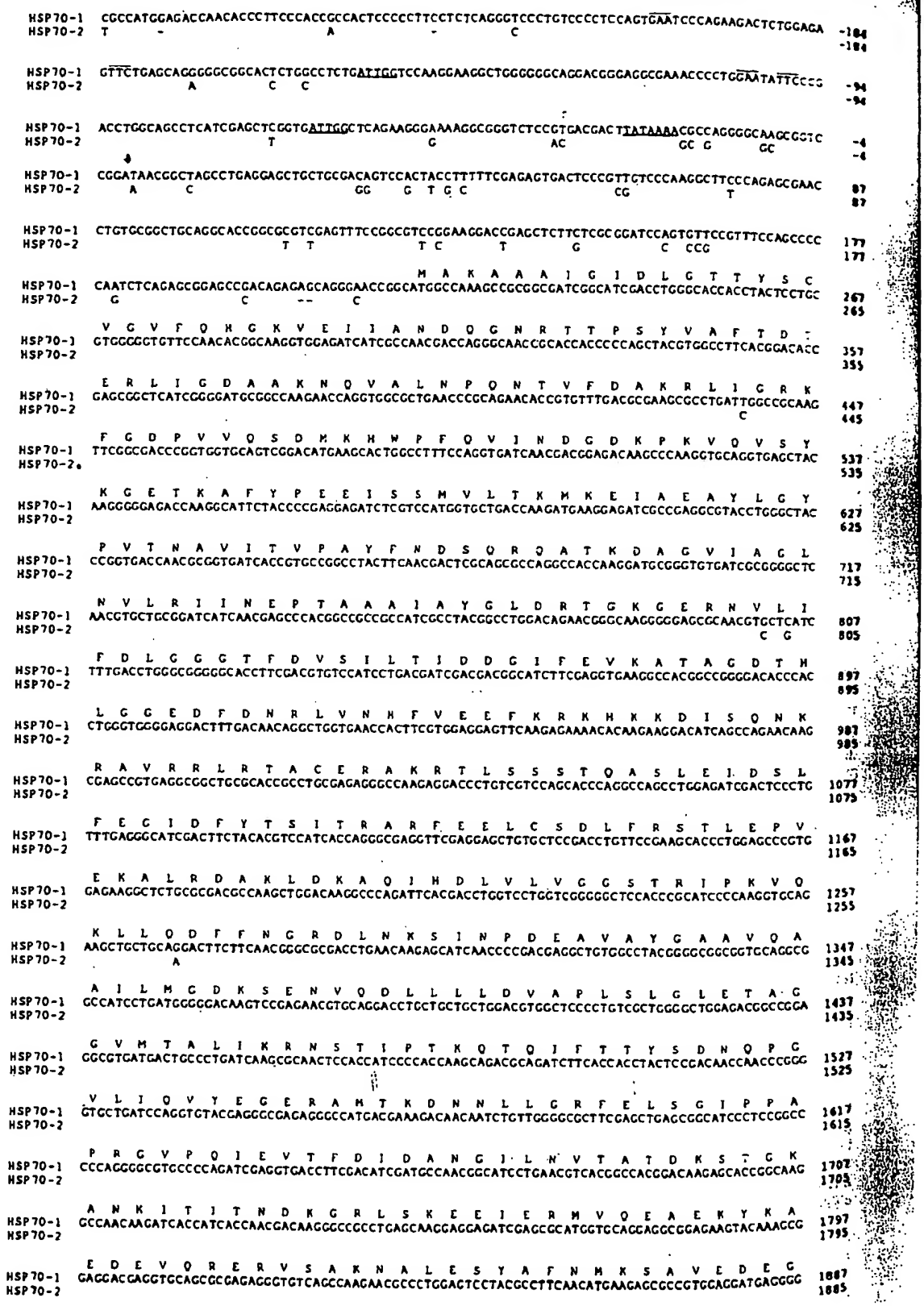


Fig. 1A. Molecular map of the MHC class III region, showing the position of the *HSP70* loci in relation to the other genes identified in this region (Sargent et al. 1989a). *Open boxes* at the *top* of the figures represent the locations of genes. The directions of transcription of the genes are shown by *arrows*, where these have been determined. The *HSP70* loci lie 92 kb telomeric to the *C2* gene. *HSP70-1* and *HSP70-2* are 11 kb apart, and the *HSP70-Hom* gene is 4 kb telomeric to *HSP70-1*. The genes *G2*, *G3*, *G5*, *G7a*, *G9*, *G9a*, and *G10* have also been designated *BAT2*, *BAT3*, *BAT4*, *BAT6*, *BAT7*, *BAT8*, and *BAT9*, respectively (Spies et al. 1989). Details of the *RD* gene and the *OSG* can be found in Levi-Strauss and co-workers (1988) and Morel and co-workers (1989). B Restriction map of that part of the MHC class III region containing the *HSP70* loci, which was isolated in overlapping cosmid clones 181 and H92. Restriction sites are shown for the enzymes *Bam* HI (M), *Bgl* II (B), *Cla* I (C), *Eco* 0109 (E), *Eco* RI (R), *Hinc* II (Hc), *Hind* III (H), *Nco* I (N), and *Pvu* II (P). The *Bam* HI site on the *right* of the map, shown by a *dotted line*, was formed by ligation of insert to vector in cos H92. The positions of the *HSP70* loci are shown by *hatched boxes* at the *top* of the figure, and the directions of transcription of the genes are indicated by *arrows*. The 0.9 kb *Bgl* II probe, H, is defined by *hatched boxes*. The *bars* at the *bottom* of the figure represent the regions cloned in pATX and Bluescript vectors.



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                 L  K  G  K  I  S  E  A  D  K  K  K  V  L  D  K  C  Q  E  V  I  S  W  L  D  A  N  T  L  A
HSP70-1  CTCAAGGGCAAGATCAGCGAGGCCGACAAGAAGAAGGTGCTGGACAAGTGTCAAGAGGTCATCTCGTGGCTGGACGCCAACACCTTGGCC  1977
HSP70-2                                                  T                                            1975

                 E  K  D  E  F  E  H  K  R  K  E  L  E  Q  V  C  N  P  I  I  S  G  L  Y  Q  G  A  G  G  P
HSP70-1  GAGAAGGACGAGTTTGAGCACAAGAGGAAGGAGCTGGAGCAGGTGTGTAACCCCATCATCAGCGGACTGTACCAGGGTGCCGGTGGTCCC  2067
HSP70-2                                                                                              2065

                 G  P  G  G  F  G  A  Q  G  P  K  G  G  S  G  S  G  P  T  I  E  E  V  D  *
HSP70-1  GGGCCTGGGGGCTTCGGGGCTCAGGGTCCCAAGGGAGGGTCTGGGTCAGGCCCCACCATTGAGGAGGTAGATTAGGGGCCTTTCCAAGAT  2157
HSP70-2            C                                     T                 G                GTTCTT  2155

HSP70-1  TGCTGTTTTTGTTTTGGAGCTTCAAGACTTTGCATTTCCTAGTATTTCTGTTTGTCAGTTCTCAATTTCCTGTGTTTGCAATGTTGAAAT  2247
HSP70-2  AGTATG   GTC    AG TGGACT TTGGGACTCAAGGACT TGC GCTG  T  CTA G   T  CTGCT CAGCT TT  C  CTTC  2245

HSP70-1  TTTTTGGTGAAGTACTGAACTTGCCTTTTTTTTCCGGTTTCTACATGCAGAGATGAATTTATACTGCCATCTTACGACTATTTCTTCTTTT  2337
HSP70-2  AC  CTT  T AAGT  T ACCTGA GG AA TA C GG  T   TATTTTTGT G AC AC GATATGT C TT GA   CT  GCA    2335

HSP70-1  TAATACACTTAACTCAGGCCATTTTTTAAGTTGGTTACTTCAAAGTAAATAAACTTTAAAATTCAAGTGATGCCCTTTTATTCCTTTATT  2427
HSP70-2     GTTGA  CTGT A GGTG   CGTTCCCTT A A GA TCAAC C GCCACC TCTG A G   TTGTTTG    T  TT   T    2425

HSP70-2  TTTTTTTTTGCTTGGCGAAAACACTACAAAGGCTGGGAATGTATGTTTTTATAATTTGTTTATTTAAATATGAAAAATAAAATGTTAAAC  2515

HSP70-2  TTTTTCTTGTCTGTTAATATGTGAAGATAATGGATATTTGCGGAGGGATAGTGTCTGAATACCATCTATCTTTATAGTCTGAAAAGAACA  2605
```

Fig. 2. Comparison of the nucleotide sequences of the *HSP70-1* and *HSP70-2* genes. The *upper line* shows the sequence of *HSP70-1* and the *lower line* shows nucleotide differences in *HSP70-2*. The transcriptional start site identified by Wu and co-workers (1985) is marked by an *arrow* and defined as position +1. The heat shock consensus elements (GAA/TTC) are indicated by *lines above* the sequence. The distal and proximal CAAT homologies (ATTGG), the TATA box (TATAAAA), and the polyadenylation signals (AATAAA) for both *HSP70-1* and *HSP70-2* are *underlined*. The stop codon (TAG) is indicated by an *asterix*. The derived protein sequence which is identical for both HSP70-1 and HSP70-2 is shown *above* the nucleotide sequence.

Hunt and Morimoto (1985). Oligonucleotides taken from sequence positions 468–489, 762–781, 1084–1101, 1342–1360, 6122–6149, and 1886–1904 (see Fig. 2) were used as sequence primers for the coding regions of both the *HSP70-1* and *HSP70-2* genes. In addition, oligos from sequence positions 2120–2137 in *HSP70-1* and positions 2118–2135 in *HSP70-2* were used to sequence the 3′ untranslated regions.

Southern blot analysis of genomic DNA. Genomic DNA (5 µg) was digested with the appropriate restriction enzyme using the conditions recommended by the supplier (Amersham, Amersham, UK). The digested DNA was fractionated on a 0.8% agarose gel, transferred to nitrocellulose (Southern 1975), and hybridized with ^{32}P-labeled probes. Probes were either labeled directly in LGT agarose, or DNA was purified using GeneClean (Siratech Scientific, London, UK) prior to labeling by random hexanucleotide priming (Feinberg and Vogelstein 1984). Blots were hybridized for 24 h at 42 °C in 50% formamide/5 × Denhardt's solution/10% dextran sulfate/1 *M* NaCl/50 m*M* Tris-HCl pH 7.4/0.1% sodium dodecyl sulfate (SDS) containing 100 µg/ml sonicated salmon sperm DNA. High-stringency washes were performed at 65 °C in 0.1 × standard sodium citrate (SSC)/0.1% SDS for 1 h. Blots were autoradiographed between two intensifying screens at −70 °C for 1–5 days.

Isolation of RNA and northern blot analysis. HeLa and U937 cells were grown in tissue culture to densities of $1–2 \times 10^6$ cells/ml. Cells were either maintained at 37 °C or heat-shocked at 42 °C for 2 h prior to their collection by centrifugation. Some HeLa cells were also fed with 2-deoxy-D-glucose at a final concentration of 10 m*M* for 12 h or starved of serum, by transfer to serum-free media for 24 h, prior to their collection. Total RNA was extracted by guanidinium isothiocyanate lysis and caesium chloride ultracentrifugation (Chirgwin et al. 1979; Maniatis et al. 1982). Polyadenylated poly(A)$^+$ mRNA was isolated from the total RNA by oligodeoxythymidylate chromatography (Maniatis et al. 1982). Samples of total RNA (15 µg) or 2 µg samples of poly(A)$^+$ RNA were fractionated in 0.8% agarose-formaldehyde denaturing gels and transferred onto nitrocellulose (Fourney et al. 1988). Northern blots were hybridized with ^{32}P-labeled probes under the same conditions as genomic Southern blots, as outlined above. High-stringency washing was carried out at 65 °C in 0.2 × SSC/0.1% SDS for 1 h, prior to autoradiography at −70 °C between intensifying screens for 2–10 days.

Results

The HSP70-1 and -2 genes. The complete sequences of the *HSP70-1* and *HSP70-2* genes and their 5′ flanking sequences were determined as described in Materials and methods. The *HSP70-1* gene has an open-reading frame of 1923 base pairs (bp) from the ATG codon at nucleotide 217 to the stop codon (TAG) at nucleotide 2140 (Fig. 2). The predicted relative mass of *HSP70-1* from the derived protein sequence (641 amino acid residues) is 70053. In the 3′ untranslated region the polyadenylation signal AATAAA lies 242 bp from the stop codon. Comparison of the sequence shown in Figure 2 with the previously published *HSP70* gene sequence (Hunt and Morimoto 1985) revealed 11 nucleotide differences. Two of these differences, a C−G transversion at nucleotide 190 and a G insertion at nucleotide 215, lie in the 5′ untranslated region. The remaining nine differences lie within the coding sequence. Of these, two single-base changes predict changes in the derived amino acid sequence at position 7 (GTC−ATC; Val−Ile) and at position 371 (GGC−GCC; Gly−Ala), in agreement with the results of Sargent and co-workers (1989b). The presence of three single-base insertions at nucleotides 1620, 1622, and 1623 in our sequence predicts an extra codon at position 1621. The sequence of nucleotides 1618–1626 in Figure 2 is 5′CCCAGGGGC3′ and encodes Pro-Arg-Gly. The sequence of Hunt and Morimoto (1985) is 5′CCAGGC3′ and encodes Pro-Gly. The additional Arg in our sequence is also found in Drosophila HSP70, *E. coli dnaK*, and HSP70B′ (Fig. 4). The differences between our sequence and that determined by Hunt and Morimoto (1985) could represent allelic polymorphism in the *HSP70-1* gene.

The *HSP70-2* sequence contains a continuous open-reading frame extending from nucleotides 215 to 2138 (Fig. 2), and like the *HSP70-1* gene lacks introns. The derived protein sequence of 641 amino acid residues is identical to that of HSP70-1. The eight nucleotide differences at positions 436, 799, 802, 1267, 1923, 2074, 2119, and 2134 (nucleotide positions refer to HSP70-2) lie in the second or third base of a codon and do not cause alteration in the derived amino acid sequence (see Fig. 2). The G→A transition at position 1267 in HSP70-2 results in the loss of a *Pst* I restriction site. This is the basis for the *Pst* I polymorphism defined by Goate and co-workers (1987). In the 3′ untranslated region of HSP70-2 the polyadenylation signal lies 361 bp from the stop codon. Comparison of the 3′ untranslated region of the *HSP70-1* gene with the equivalent region of the *HSP70-2* gene reveals that they are completely divergent (Fig. 2).

The 5′ untranslated region of the *HSP70-1* gene is 217 bp and is identical to that determined by Hunt and Morimoto (1985) except for the two single-base changes mentioned above. Although the transcriptional start site of the *HSP70-2* gene has not been determined, it is likely that it is at a similar position to that of the *HSP70-1* gene. In primer extension experiments reported by others (Wu et al. 1985), only a single extended product was defined. Given that both genes are transcribed into abundant mRNA species after heat shock (Fig. 6) and that the two sequences differ by only 26 single-base changes, any primer extension based on *HSP70-1* mRNA with the primers used would also have occurred on *HSP70-2* mRNA. In addition, this would place the TATA box sequence at the appropriate place in the *HSP70-2* gene from the transcriptional start site.

Comparison of the 5′ flanking sequence of the *HSP70-1* and -2 genes reveals an additional 17 single-base changes extending back as far as nucleotide -273. However, none of the differences seriously disrupt any of the elements so far identified in the *HSP70-1* gene as being important for the basal level of transcription of the gene, or for transcriptional activation after stress (Wu et al. 1986; Morgan et al. 1987; Amin et al. 1988; Williams et al. 1989; Taylor and Kingston 1990).

The homologous region. The 0.9 kb *Bgl* II probe (H), derived from the *HSP70-1* gene, in addition to hybridizing to the *HSP70-1* and *HSP70-2* genes on Southern blot analysis, was found to hybridize less strongly to a region lying −4 kb telomeric to the *HSP70-1* gene (Sargent et al. 1989b). In order to define the structure of the homologous region the sequence of 3330 bp of DNA was determined (Fig. 3). This indicated that the homologous region represents another gene (*HSP70-Hom*) of the *HSP70* family. The *HSP70-Hom* gene contains an open-reading frame of 1923 bp from the ATG codon at nucleotide 960 to the stop codon (TAA) at nucleotide 2883. Like the *HSP70-1* and -2 genes, it lacks introns. The 3′ untranslated sequence contains a polyadenylation signal ATTAAA 405 bp from the stop codon and differs significantly from that of the *HSP70-1* and -2 genes. Upstream of the ATG codon at nucleotide 960 are two Alu repeat sequences between nucleotides 294 and 900. A TATA box is found at nucleotide 162. However, no CAAT box sequence or heat shock consensus element with GAA/TTC repeats is found up to 959 bp from the ATG codon.

Comparison of the amino acid sequences of HSP70-Hom and HSP70-1 (Fig. 4) reveals that they are 90% similar. If conservative replacements are taken into account the sequence similarity increases to 94%. The greatest sequence divergence between these two proteins is at the C terminus. The similarity in the C-terminal 100 amino acids is only 72%. There is also a high level of protein sequence identity between HSP70-Hom and HSP70-B′ (79%; Leung et al. 1990) and between HSP70-Hom and HSC70 (84%; Dworniczak and Mirault 1987). Again the sequences differ most in the C-terminal 100 amino acids, where the percentage similarity decreases to 47% between HSP70-Hom and HSP70B′, and to 64% between HSP70-Hom and HSC70.

Southern blot analysis. The human genome contains multiple copies of expressed genes and unexpressed pseudogenes in the *HSP70* family. Due to the high degree of sequence similarity between the coding regions of the *HSP70* genes, DNA probes corresponding to coding regions tend to cross-hybridize to each other. This is apparent in Figure 5A where probe H, in addition to hybridizing to the 2.4 kb, 5 kb, and 7 kb *Bam* HI/*Hind* III fragments which correspond to the *HSP70-1*, *HSP70-Hom*, and *HSP70-2* genes, respectively, also hybridized to other fragments corresponding to *HSP70* genes elsewhere in the genome. In order to differentiate between the various genes in genomic Southern blot analysis and to provide evidence for the expression of the MHC-linked *HSP70* genes in northern blot analysis, it was necessary to define probes corresponding to the regions of minimum sequence similarity between the various genes. Given that the 3′ untranslated regions of each of the three genes showed little sequence similarity to each other or to the 3′ untranslated regions of the *HSP70B′* (Leung et al. 1990) and *HSC70* (Dworniczak and Mirault 1987) genes, the isolation of suitable locus-specific probes was possible.

A 510 bp *Eco* 0109/*Hind* III fragment, a 600 bp *Eco* 0109 fragment, and a 225 bp *Eco* 0109 fragment were isolated from the 3′ untranslated regions of the *HSP70-1*, *HSP70-2*, and *HSP70-Hom* genes, respectively. Each of these probes was hybridized to a Southern blot of genomic

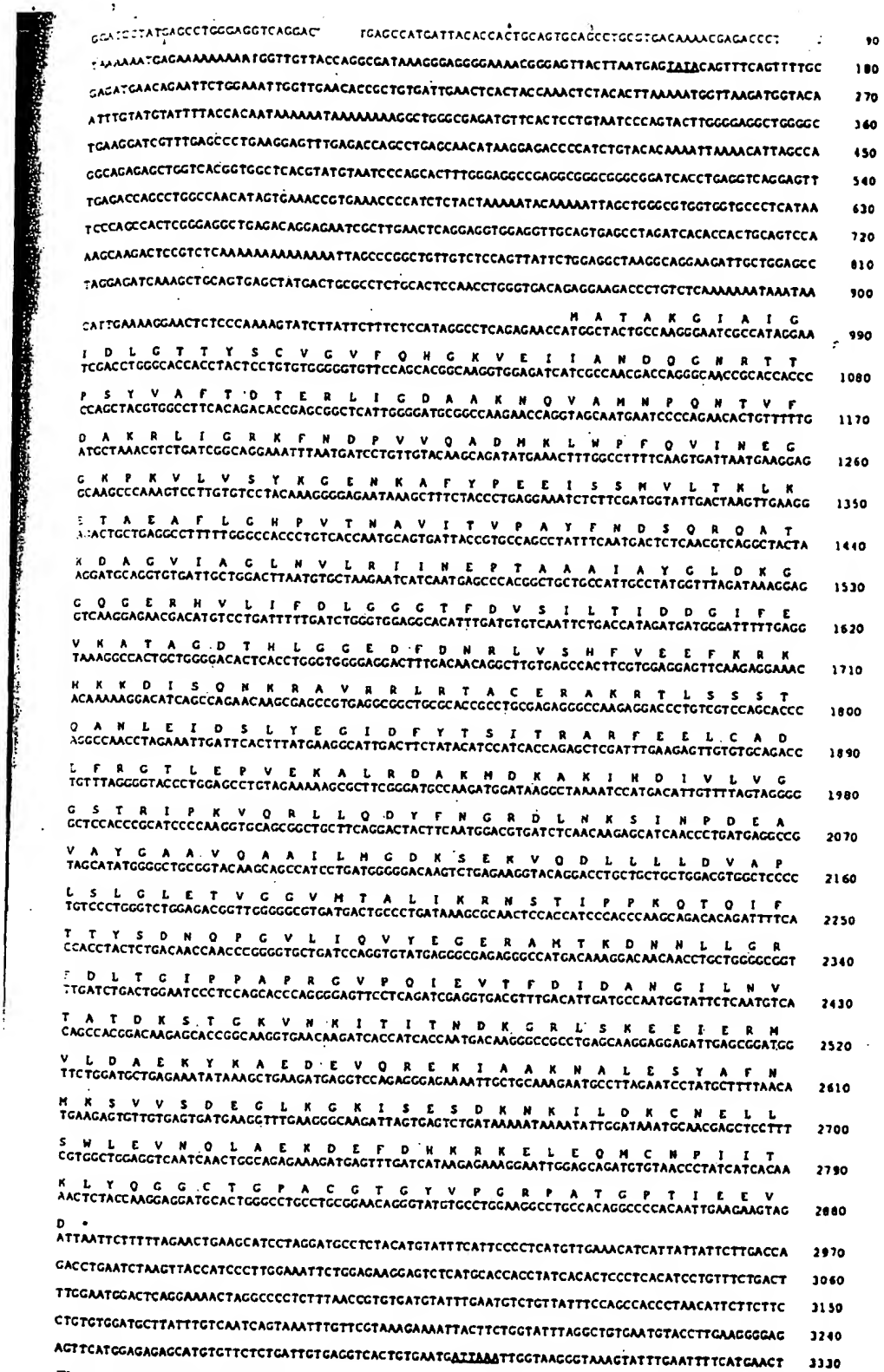

Fig. 3. Sequence of the *HSP70-Hom* gene. The TATA box and polyadenylation signal (ATTAAA) are *underlined*. The stop codon (TAA) is indicated by an *asterix*. The derived protein sequence is shown *above* the nucleotide sequence.

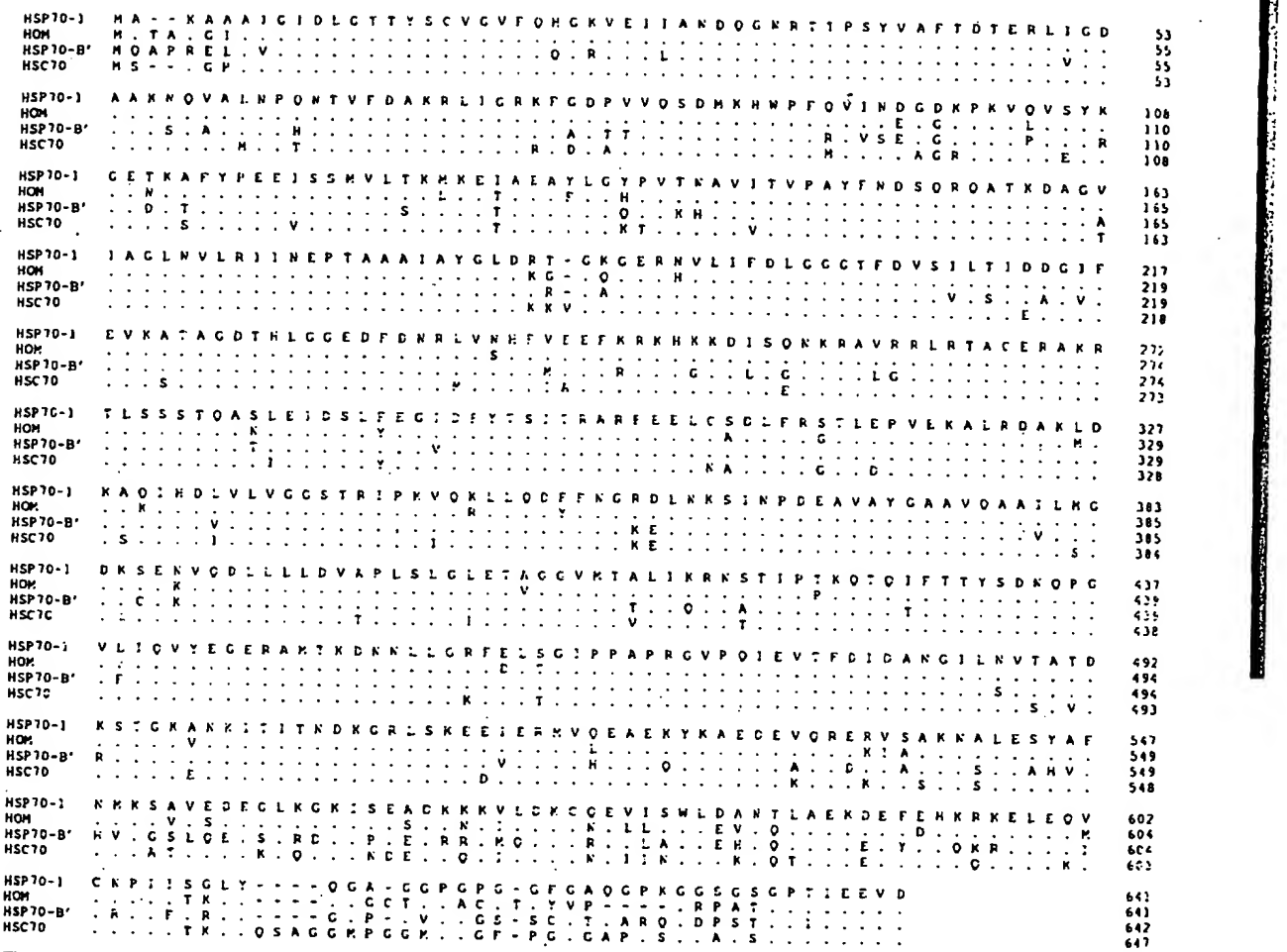

Fig. 4. Comparison of the protein sequences of HSP70-1 and HSP70-Hom with those of the stress-induced protein HSP70-B' (Leung et al. 1990) and the constitutive protein HSC70 (Dworniczak and Mirault 1987). Amino acid residues that differ from HSP70-1 are shown and identical amino acids are indicated by a *dot*. *Dashes* are inserted to optimize alignment of the protein sequences.

DNA digested with *Eco* RI, *Bgl* II, *Bam* HI, and *Hind* III in single- and double-digest combinations. In all cases the probes hybridized uniquely to fragments associated with the *HSP70* locus from which they had been isolated, and to no other fragments (Fig. 5B).

Northern blot analysis. The locus-specific probes described above were used to examine the expression of the MHC-linked *HSP70* genes. Northern blots were prepared using total RNA from HeLa cells either maintained at 37 °C, heat-shocked for 2 h at 42 °C, fed with 2-deoxy-D-glucose for 12 h, or serum-starved for 24 h prior to harvesting. In addition, poly(A)$^+$ RNA from control and heat-shocked U937 cells was used.

With both the *HSP70-1* and *HSP70-2* locus-specific probes an elevated level of a ~2.4 kb mRNA was observed from the heat-shocked HeLa (Fig. 6) and U937 cells (data not shown). A very low level of *HSP70-1* mRNA was detectable in the control cells, but this was not observed for *HSP70-2* (Fig. 6). However, neither 2-deoxy-D-glucose treatment nor serum starvation had any apparent effect on the level of message observed constitutively, in agreement with the results of others (Wu and Morimoto 1985; Wu et al. 1985; Watowich and Morimoto 1988). Assuming that the *HSP70-1* and *HSP70-2* genes share the same transcriptional start site (defined by Wu et al. 1985), the positions of the polyadenylation signals in these two genes predict an mRNA for *HSP70-2* ~100 bp longer than that for *HSP70-1*. The resolution available by northern blotting is not sufficient for this difference to be seen.

When the *HSP70-Hom* locus-specific probe was used to probe northern blots a very low level of an mRNA of ~3 kb was detected from control HeLa cells (Fig. 6) and also heat-shocked, 2-deoxy-D-glucose-treated, and serum-starved cells, a result which indicates that this gene is

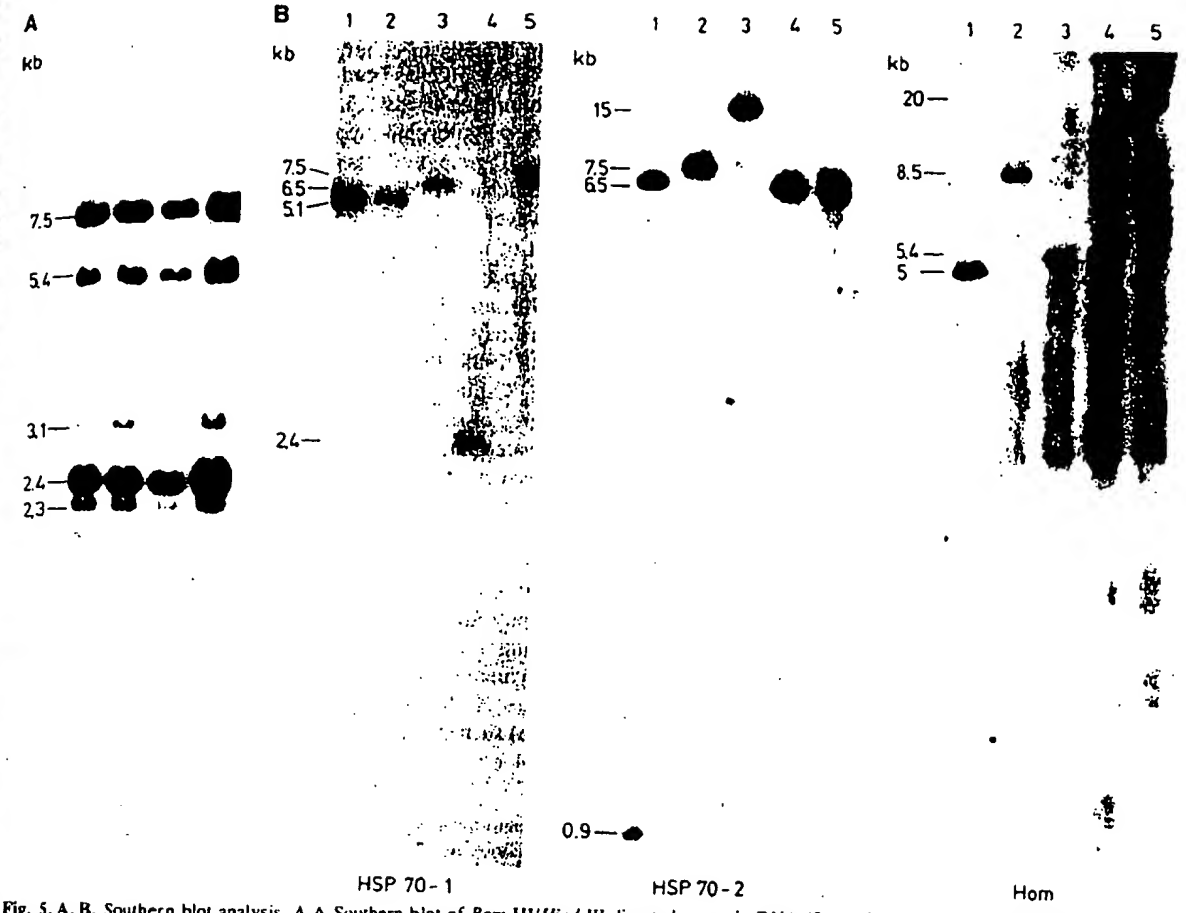

Fig. 5. A, B. Southern blot analysis. A A Southern blot of *Bam* HI/*Hind* III-digested genomic DNA (5 μg) from four individuals was hybridized with the ^{32}P-labeled 0.9 kb *Bgl* II probe (H). After high-stringency washing and autoradiography the probe was found to hybridize strongly to fragments of 7.5, 5.4, and 2.4 kb, which correspond to the MHC-linked *HSP70* loci. In addition, probe H hybridized less strongly to fragments of 3.1 and 2.3 kb, associated with *HSP70* loci elsewhere in the genome. B Locus-specific probes for the MHC-linked *HSP70* loci. Southern blots of genomic DNA (5 μg) from an *HLA*-homozygous cell line (ICE 5) digested with *Eco* RI/*Bgl* II (1), *Bgl* II (2), *Hind* III (3), *Bam* HI/*Hind* III (4), and *Bam* HI (5) were hybridized with the ^{32}P-labeled locus-specific probes prepared from the 3′ untranslated regions of the MHC-linked *HSP70* genes. After high-stringency washing and autoradiography, each of the probes hybridized only to fragments shown by cosmid mapping to be associated with the genes from which they had been derived. Sizes of fragments are shown on the *left* of each blot.

expressed constitutively at a low level but is not induced by heat shock, 2-deoxy-D-glucose, or serum starvation. Although the transcriptional start site of the homologous gene has not been determined, an mRNA of ~3 kb is consistent with the positions of the TATA box (nucleotide 162) and polyadenylation signal (nucleotide 3290).

Discussion

Our analysis of the MHC-linked *HSP70* loci has shown the presence of two intronless genes, *HSP70-1* and *HSP70-2*, which encode an identical protein product and which both express elevated levels of a ~2.4 kb mRNA following heat shock. The observation that two closely linked *HSP70* loci both express an identical protein is somewhat surprising. One possible explanation is that the range of stress factors which induce HSP70 expression is too great for the promotor sequence of a single gene to recognize them all. Therefore, two genes with different 5′ promotor sequences, as in the case of *HSP70-1* and *HSP70-2*, are required to provide an adequate response. In addition, there may be differences in the expression of the two genes in different cell types due to the sequence differences in the promoter regions. Also, the sequences in the 3′ untranslated region which are completely divergent between *HSP70-1* and *HSP70-2* may confer different regulation on the two mRNA species.

Lying ~4 kb telomeric of *HSP70-1* is a third intronless gene, *HSP70-Hom*, which encodes a protein with 90% similarity to HSP70-1. The *HSP70-Hom* gene appears to be constitutively expressed, but levels of its mRNA are not increased following heat shock. This novel

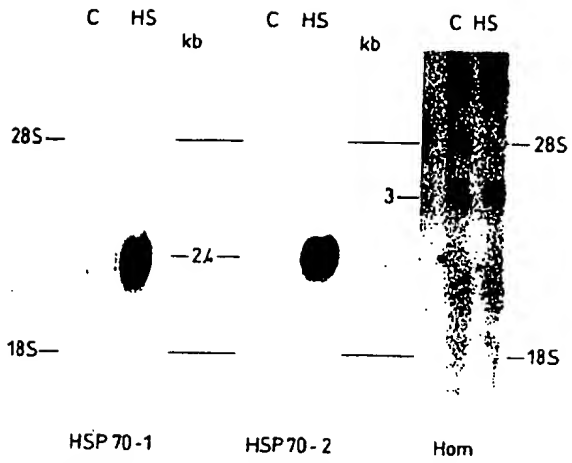

Fig. 6. Northern blot analysis. Total cytoplasmic RNA (15 μg) derived from HeLa cells, either maintained at 37 °C or heat-shocked for 2 h at 42 °C prior to harvesting, was fractionated on 1% agarose-formaldehyde denaturing gels and transferred onto nitrocellulose to give three identical blots. The northern blots were hybridized with the three *HSP70* locus-specific probes. The position of migration of the 28S and 18S RNA is shown. The *HSP70-1* probe detected a transcript of ~2.4 kb at a very low level in constitutive RNA (C) and at a much elevated level in heat-shocked RNA (HS). The *HSP70-2* probe detected no transcript in constitutive RNA (C), and a high level of a ~2.4 kb transcript in heat-shocked RNA (HS). The homologous gene probe detected low levels of a ~3 kb transcript in constitutive (C) and heat-shocked (HS) RNA.

HSP70 gene encodes an HSP70 variant that is more basic than HSP70-1, and is distinct from HSC70 and the recently described HSP70B′ (Leung et al. 1990), which are encoded by genes in other parts of the genome.

Comparison of HSP70-1 and HSP70-Hom with other members of the HSP70 protein family reveals that they are highly similar (79%–84%). The greatest divergence is in the C-terminal 100 amino acids (HSP70-Hom vs HSP70-1, 72%; HSP70-Hom vs HSP70B′, 47%; HSP70-Hom vs HSC70, 64%) and this may indicate a selective function of the different HSP70 proteins.

Transcription of the *HSP70-1* gene has been shown to be induced by serum stimulation after serum starvation (Wu and Morimoto 1985). However, serum withdrawal alone has been shown to result in increased levels of a cytosolic protein, prp 73, in rats (Chiang et al. 1989). This protein has been shown to be a member of the HSP70 family and has been found to bind to peptide regions that target intracellular proteins for lysozomal degradation, in response to serum withdrawal. We therefore looked for elevated levels of mRNA from the MHC-linked *HSP70* genes following serum withdrawal, but this was not observed. Elevated levels of mRNA from the glucose-responsive *HSP70* family member *GRP78* (*BiP*) have been shown to occur following treatment with 2-deoxy-D-glucose (Watowich and Morimoto 1988). However, elevated levels of mRNA were not detected for any of the MHC-linked *HSP70*, following 2-deoxy-D-glucose treatment of cells in culture.

The MHC has been shown to influence more than 40 autoimmune diseases. In some cases, a direct involvement of the polymorphic class I and class II and the less variable class III gene products has been proposed (Batchelor and McMichael 1987; Todd et al. 1988). In many cases, however, the role of the MHC is unclear. There is a great deal of interest in the possible roles that stress proteins may play in immune responses (for review see Young 1990), and there is evidence to suggest that HSP70 proteins may be involved in autoimmune diseases (van Eden et al. 1988; Res et al. 1988; Lamb et al. 1989; Yasuhiro and Kishimoro 1990). However, due to the very high degree of sequence similarity it is not easy to distinguish between the different HSP70 family members using cDNA or genomic probes. Thus, the locus-specific probes reported here will make it possible to investigate variations in gene copy number and variations in the levels of expression of the MHC-linked *HSP70* genes in relation to those *HLA* types frequently associated with autoimmunity. In addition, the sequence differences in the 5′ and 3′ untranslated regions allow the specific amplification by polymerase chain reaction of the MHC-linked *HSP70* genes (C. M. Milner and R. D. Campbell, unpublished data) such that sequence variation in these genes between haplotypes can be investigated.

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